THE BIOLOGY OF DEATH. II—CONDITIONS OF CELLULAR IMMORTALITY[1]

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1. Artificial Parthenogenesis

IN the preceding paper in this series it was pointed out that the germ cells of higher organisms are potentially, and under certain conditions in fact, immortal. What are the conditions of immortality in this case? Are they such as to support the thesis that the processes of mortality are essentially physico-chemical in nature, and follow physico-chemical laws?

The most essential condition of this immortality of germ cells was mentioned, but not particularly emphasized. It is that two germ cells, an ovum and a spermatozoon *unite*, the process of union being called fertilization. Having united, if they then find themselves in appropriate environmental conditions, development goes on, new germ cells and a soma are formed, and the same process keeps up generation after generation. Now while union of the germ cells is generally and in most organisms an essential condition of this process, it is also true that in a few forms of animal life, mostly found among the invertebrates, development of the ovum can take place without any preceding fertilization by a spermatozoon. The process of reproduction in this case is called *parthenogenesis*. In a number of forms in which parthenogenesis never occurs normally, so far as is known, it can be induced by appropriate extraneous procedures. The discovery of this extraordinarily interesting and important fact for a number of organisms, and the careful working out of its physico-chemical basis, we owe to Dr. Jacques Loeb, of the Rockefeller Institute for Medical Research. Artificial parthenogenesis may be induced, as Guyer, Bataillon and Loeb have shown in so highly organized a creature even as the frog, and the animal may grow to full size. The frogs shown in Figure 1, while they present much the same appearance as any other frog of the same species, differ in the rather fundamentally important respect that they had no father.

The rôle of a father was played in these cases by an ordinary dissecting needle. Unfertilized eggs from a virgin female were gently pricked on the surface with a sharply pointed needle. This initiation

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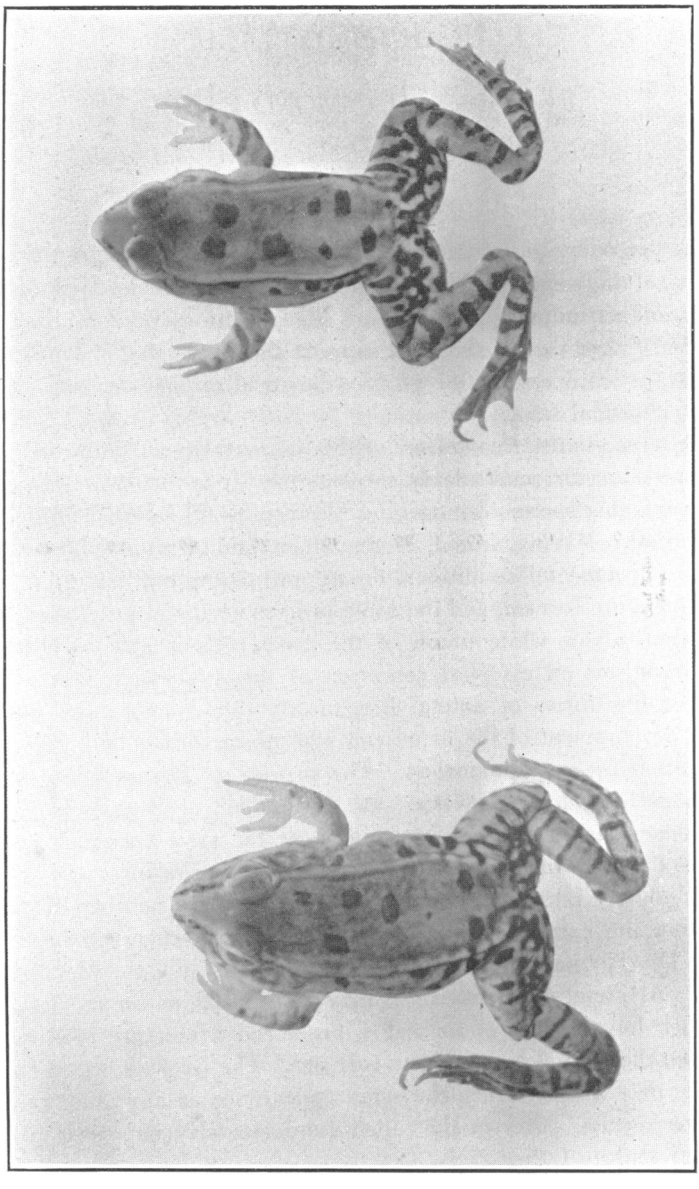

FIG. 1. ARTIFICIALLY PARTHENOGENETIC FROGS. (Loeb.)

of the process of development took place March 16, 1916, in one case, and February 27, 1917 in the other. The date of death was in the first case May 22, 1917 and in the other March 24, 1918.

In the course of Loeb's studies of parthenogenesis in lower marine invertebrates, he became interested in the question of the death of the germ cells which had failed to unite, or having united failed of appropriate environmental conditions. His researches throw light on some of the conditions of cellular death, and on that account they may be reviewed briefly here. He found that the unfertilized mature eggs of the sea-urchin die comparatively soon when deposited in sea-water. The same eggs, however, live much longer, and will if appropriate surrounding conditions are provided go on and develop an adult organism, if they are caused to develop artificially by chemical means or naturally by fertilization. Loeb concluded from this that there are two processes going on in the egg. He maintained, on the one hand, that there are specific processes leading to death and disintegration, and, on the other hand, processes which lead to cell division and further development. The latter processes may be regarded as inhibiting or modifying the mortal process. Loeb and Lewis undertook experiments based upon this view to see whether it would be possible by chemical treatment of the egg to prolong its life. Since in general specific life phenomena are perhaps on the chemical side chiefly catalytic phenomena, it was held to be reasonable that if some substance could be brought to act on the egg, which would inhibit such phenomena without permanently altering the constitution of the living material the life of the cell should be considerably prolonged. The first agent chosen for trial was potassium cyanide, KCN. It was known that this substance weakened or inhibited entirely a number of enzymatic processes in living material, without materially or permanently altering its structure.

It was found that normally the unfertilized egg of the sea-urchin would live in sea-water at room temperature, and maintain itself in condition for successful fertilization and development, up to a period of about twenty-three hours. After that time the eggs began to weaken. Either they could not be successfully fertilized, or, if they were fertilized, development only went on for a short time. After 32 hours the eggs could not as a rule be fertilized at all. The experiment was then tried of adding to the sea-water, in which the unfertilized eggs were kept, small amounts of KCN in a graded series, and then examining the results of fertilizations undertaken after a stay of the unfertilized eggs of 75 hours in the solution. It will be noted that this period of 75 hours is more than three times the normal duration of life of the cell in normal sea-water. The results of this experiment are shown in summary form in Table 1.

TABLE I

Experiments of Loeb and Lewis on the prolongation of life of the sea-urchin egg by KCN

Concentration of KCN	Result of fertilization after a 75 hours' stay in the solution
Pure sea-water	No egg segments
n/64000 KCN	No egg segments
n/16000 KCN	No egg segments
n/8000 KCN	Very few eggs show a beginning of segmentation
n/4000 KCN	Very few eggs show a beginning of segmentation
n/2000 KCN	Few eggs go through the early stages of segmentation
n/1000 KCN	Many eggs segment and develop into swimming larvae
n/750 KCN	Many eggs segment and develop into swimming larvae
n/400 KCN	A few eggs develop into swimming larvae
n/300 KCN	No egg segments
n/250 KCN	No egg segments
n/200 KCN	No egg segments
n/100 KCN	No egg segments

From this table it is seen that in concentrations of KCN from n/750 to n/1000 the eggs developed perfectly into swimming larvae. In other words, by the addition of this very small amount of KCN the life period has been prolonged to three times what it would normally be under the same environmental conditions. Concentrations of KCN weaker than n/1000 were incapable of producing this result, or at best if development started the process came very quickly to an end. In stronger concentrations than n/400 the eggs were evidently poisoned, and no development occurred.

Other experiments of Loeb's show that the lethal effects of various toxic agents upon the egg cell may be inhibited, or postponed for a relatively long time, by suitable chemical treatment, such as lack of oxygen, KCN, or chloral hydrate. A typical experiment of this kind made upon the sea-urchin, *Strongylocentrotus purpuratus*, may be quoted:

> Eggs were fertilized with sperm and put eleven minutes later into three flasks, each of which contained 100 c. c. of sea-water + 16 c. c. 2-1/2 m $CaCl_2$. One flask was in contact with air, while the other two flasks were connected with a hydrogen generator. The air was driven out from these two flasks before the beginning of the experiment. The eggs were transferred from one of these flasks after four hours and fourteen minutes, from the second flask after five hours and twenty-nine minutes, into normal (aerated) sea-water. The eggs that had been in the hypertonic sea-water exposed to air were transferred simultaneously with the others into separate dishes with aerated normal sea-water. The result was most striking. Those eggs that had been in the hypertonic sea-water with air were all completely disintegrated by "black cytolysis." Ten per cent. of the eggs had been transformed into "shadows" (white cytolysis). It goes without saying that all the eggs that had been in the aerated hypertonic sea-water five and a half hours were also dead. The eggs that had been in the same solution in the absence of oxygen appeared all normal when they were taken out of the solution, and three hours later—the temperature was only 15°C—they were all, without exception in a perfectly normal two- or four-cell stage. The further development was also in most cases normal. They swam as larvae

at the surface of the vessel and went on the third day (at the right time) into a perfectly normal pluteus stage, after which their observation was discontinued. Of the eggs that had been five and a half hours in the hypertonic sea-water deprived of oxygen, about 90 per cent. segmented.

Let us consider one more illustration from Loeb's work in this field. Normally, in the forms with which he chiefly worked, sea-urchin, starfish, and certain molluscs, an absolutely essential condition for the continuation of life of the germ-cells after they are discharged from the body is that two cells, the ovum and the spermatozoon, shall unite in normal fertilization. Put in another way, parthenogenesis does not normally occur in these forms. Fertilization is an essential condition for the continuation of life and development. But Loeb's painstaking and brilliant researches extending over a number of years show that when we say that fertilization is an essential condition for the continued life of the germ-cells outside the body our language tends to obscure the most important fact, which is simply that for the continuation of life in these cells only certain internal physico-chemical conditions and adjustments must be realized. It makes no essential difference to the result whether these conditions are realized through the intervention of the sperm, as in normal fertilization, or by purely artificial chemical methods initiated, controlled and directed at every step by human agency. We can, in other words, regard all cases of successful artificial parthenogenesis as fundamentally a contribution to the physiology of natural death, and a demonstration of its essentially mechanistic basis. The conditions of continued existence are physical and chemical and controllable as such. The methods finally worked out as optimum are very neat, and afford a complete demonstration of the thesis we have just stated. Thus, for example, the unfertilized egg of the sea-urchin, *Strongylocentrotus purpuratus*, will continue in life and develop perfectly normally if it is subjected to the following treatment: The eggs are first placed in sea-water to which a definite amount of weak solution of butyric acid has been added (50 c. c. of sea-water+2.8 c. c. n/10 butyric acid). In this solution at 15°C. the eggs are allowed to remain from 1½ to 3 or 4 minutes. They are then transferred to normal sea-water, in which they remain from 15 to 20 minutes. They are then transferred for 30 to 60 minutes at 15°C. to sea-water which has had its osmotic pressure raised by the addition of some salts (50 c. c. of sea-water + 8 c. c. of 2½ m NaCl, or 2½ m NaCl + KCl + $CaCl_2$ in the proportion in which these salts exist in sea-water). After the stay of from 30 to 60 minutes in this solution the eggs are transferred back to normal sea-water, the transfer being in batches at intervals of 3 to 5 minutes between each batch transferred. It is then found that those eggs which have been just the right length of time in the hypertonic sea-water develop into perfectly normal sea-urchin larvae. In other words, we have here a definite and know physico-chemical process com-

pletely replacing what was before this work universally regarded as a peculiarly vital process of extraordinary complexity, probably beyond powers of human control.

These three examples from Loeb's work on the subject of prolongation of life in the egg cell will suffice for our present purposes. The lesson which they teach is plain, and is one which has, as will be readily perceived, a most important bearing upon the general concept of life and death outlined in the preceding paper in this series. The experiments demonstrate that the conditions essential to continued life of the germ-cells outside the body are physico-chemical conditions, and that when these cells die it is because the normal physico-chemical machinery for the continuation of life has either broken down, or has not been given the proper activating chemical conditions.

Lack of space alone prevents going in detail into another extremely interesting and important development of this subject due to Dr. Frank R. Lillie of the University of Chicago. He has in recent years made a thorough analysis of the biological factors operating when the egg of the sea-urchin is normally fertilized by a spermatozoon. The conception of the process of fertilization to which Lillie comes is "that a substance borne by the egg (fertilizin) exerts two kinds of actions, (1) an agglutinating action on the spermatozoon and (2) an activating action on the egg. In other words, the spermatozoon is conceived, by means of a substance which it bears and which enters into union with the fertilizin of the egg, to release the activity of this substance within the egg." From the standpoint of the present discussion it is obvious that Lillie's results present nothing which in any way disturbs the conclusion we have reached as to the essentially physico-chemical nature of the processes which condition the continuation of life and development of the egg.

2. Tissue Culture in Vitro

Let us turn now to another question. Are the germ-cells the only cells of the metazoan body which possess the characteristic of potential immortality? There is now an abundance of evidence that such is not the case, but that on the contrary there are a number of cells and tissues of the body, which under appropriate conditions may continue living indefinitely, except for the purely accidental intervention of lethal circumstances. Every childs knows that all the tissues do not die at the same time. It is proverbial that the tail of the snake, whose head and body have been battered and crushed until even the small boy is willing to admit that the job of killing is complete, will not die till the sun goes down. Galvani's famous experiment with the frog's legs only succeeded because some parts survive after the death of the organism as a whole. As Harrison points out "Almost the whole of our knowledge of muscle-nerve physiology, and much of that of the action of the heart, is based

upon experiments with surviving organs, and in surgery, where we have to do with changes involved in the repair of injured parts, including processes of growth and differentiation, the power of survival of tissues and organs and their transplantability to strange regions, even to other individuals, has long formed the basis of practical procedures."

The first successful cultures of somatic cells and tissues outside the body were those of Leo Loeb, described in 1897. His first method consisted in cultivating the tissues in appropriate media in test tubes. Later he used also another method which involved the transplantation of the solid medium and the tissue into the body of another animal. What has been regarded as a defect of both these methods is that they do not permit the continued observation of the cells of the growing cultured tissue. To Harrison is due the development of a method which does permit such study. In 1907 he announced the discovery that if pieces of the developing nervous system of a frog embryo were removed from the body with fine needles, under strictly aseptic precautions, and placed on a sterile cover slip in a drop of frog lymph, and the cover slip then inverted over a hollow glass slide, that the tissues would remain alive for many days, grow and exhibit remarkable transformations. By this technique it was possible to study the changes with a high power of the microscope and photograph them.

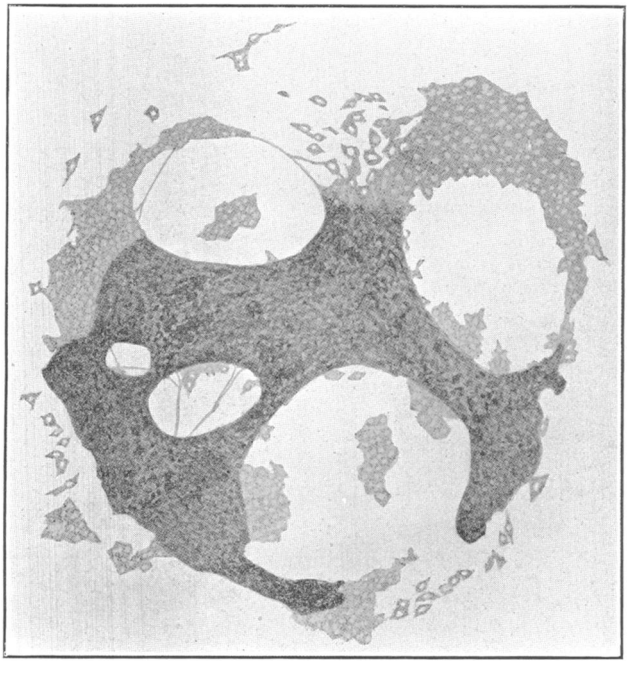

FIG. 2. PIECE OF TISSUE FROM FROG EMBRYO CULTIVATED IN LYMPH, 2 days old. The dark portion shows original bit of tissue. Lighter portions are new growth. (From Harrison.)

Figure 2 is a general view of one of these tissue cultures two days old. It shows a piece of nervous tissue from the frog embryo with cells growing out from it into the lymph. The lighter portions are the new cells. In his remarkable monograph Harrison shows nerve cells developing fibers at first thickened, but presently becoming of normal character and size. At the ends are pseudopodial processes, by which the growing fiber attaches itself to the cover slip or other solid bodies and pulls itself out, as it were. Figure 3 shows a particularly beautiful nerve fiber preparation made by Burrows.

The fibers grew from a preparation of the embryonic nervous system of the chick. There can be no doubt, as these figures so clearly show, of the life of these cells outside the body, or of the normality of their developmental and growth processes.

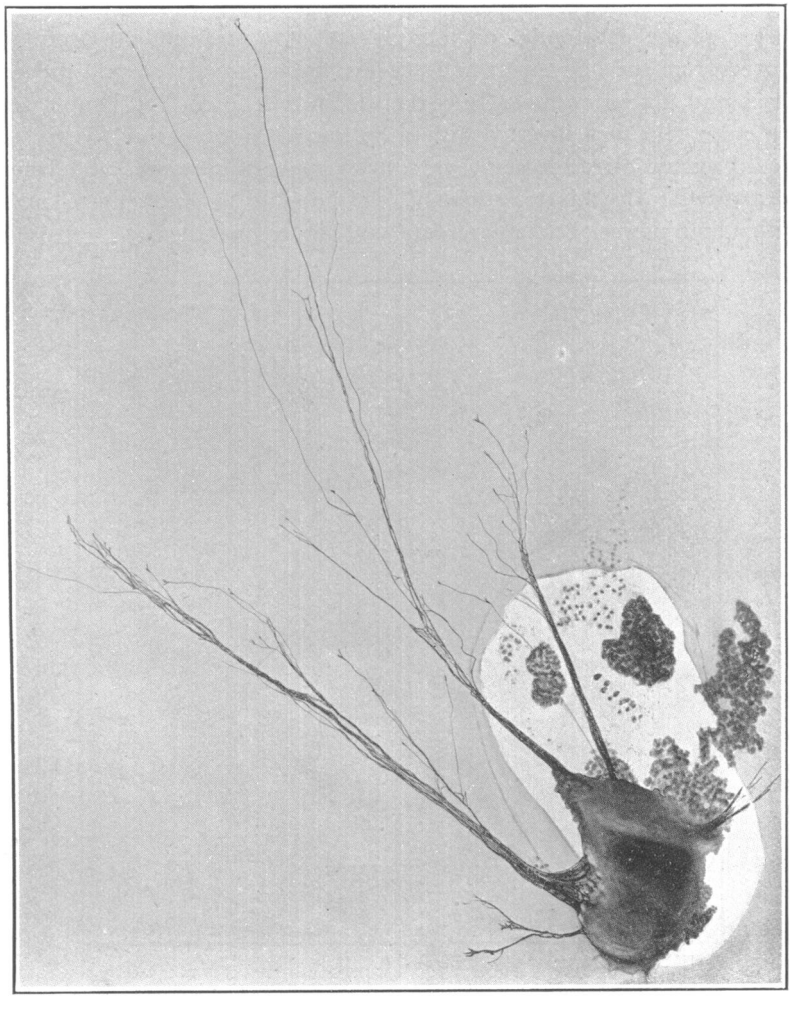

FIG. 3. GROUP OF NERVE FIBERS WHICH HAVE GROWN FROM AN ISOLATED PIECE OF NEURAL TUBE OF A CHICK EMBRYO. (From Harrison after Burrows.)

Under the guidance of Harrison, one of his students, Burrows, improved the technique of the cultivation of tissues outside the body, first by using plasma from the blood instead of lymph and later in various other ways. He devised an apparatus for affording the tissue culture a continuous supply of fresh nutrient medium. There is in this apparatus a large culture chamber which takes the place of the plain hanging drop in a hermetically sealed cell. On the top of this culture chamber there is a wick which carries the culture fluid from a supplying chamber and discharges it into a receiving chamber. The tissue is planted among the fibers of the wick, which are pulled apart where it crosses the top of the chamber. The whole system is kept sterile and so arranged that the growing tissue can be kept under observation with high powers of the microscope. The nutrient medium may be modified at will, and the effects of known substances upon the cellular activities of every sort may be studied.

Burrows began his investigations in this field on the tissues of the embryo chick. With the success of these cultures was established the fact that the tissues of a warm blooded animal were as capable of life, development, and growth outside the body as were those of cold-blooded animals, such as the frog. Burrows succeeded in cultivating outside the body cells of the central nervous system, the heart, and mesenchymatous tissue of the chick embryo. At the same time Carrel was carrying on studies in this same direction at the Rockefeller Institute. In his laboratory were made the first successful cultures in vitro of the adult tissue of mammals. He developed a method of culture on a plate which permitted the growing of large quantities of material. He found that almost all the adult and embryonic tissues of dog, cat, chicken, rat, guinea pig, and man could be cultivated *in vitro*. Figure 4 shows a culture of human tissue, made at the Rockefeller Institute. I am indebted to Doctor Carrel and Doctor Ebeling for permission to present this photograph here.

According to the nature of the tissues cultivated, connective or epithelial cells were generated, which grew out into the plasma medium in continuous layers or radiating chains. Not only could normal tissues be cultivated but also the cells of pathological growths (cancer cells). It has been repeatedly demonstrated that normal cell division takes place in these tissues cultivated outside the body. The complex process of cell division which is technically called mitosis, has been rightly regarded as one of the most characteristic, because complicated and unique, phenomena of normal life processes. Yet this process occurs with perfect normality in cells cultivated outside the body. Tissues from various organs of the body have been successfully cultivated, including the kidney, the spleen, the thyroid gland, etc. Burrows was even able to demonstrate that the isolated heart muscle cells

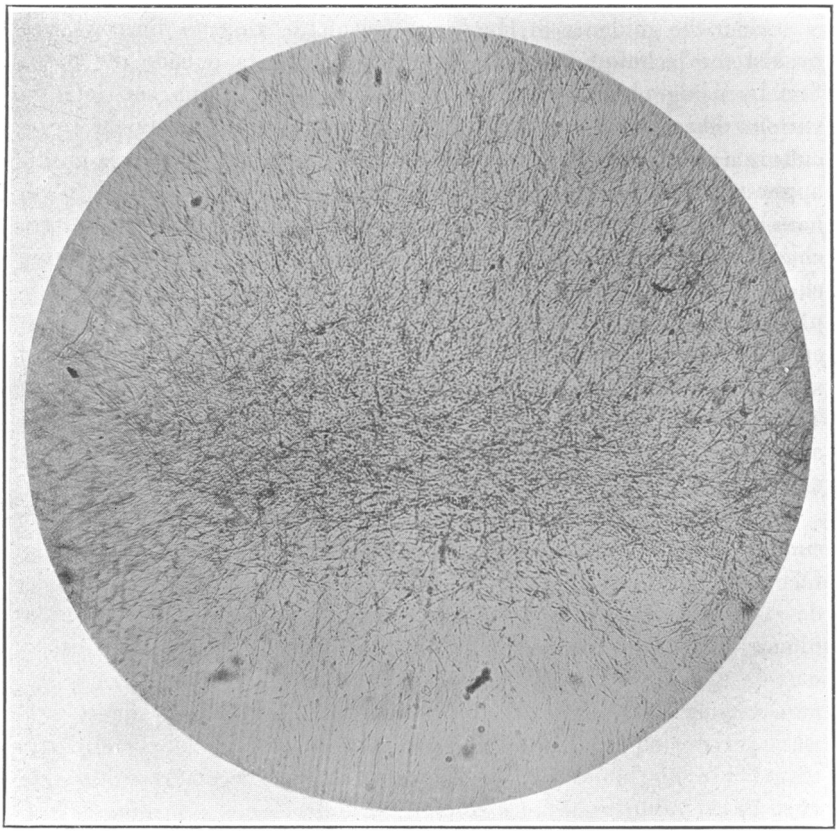

FIG. 4. HUMAN CONNECTIVE TISSUE CELLS FIXED AND STAINED WITH GIEMSA STAIN. The culture was made by extirpating the central portion of culture 285 in its 16th passage, washing the remaining portion of the culture with Ringer solution without removing it from the cover-glass, and dropping on fresh plasma and extract. The preparation shows the extent of growth obtained in 48 hours from peripheral cells remaining after extirpation of the fragment. (After Losee and Ebeling.)

of the chick embryo can divide as well as differentiate and beat *rhythmically* in the culture medium.

Perhaps even more remarkable than the occurrence of such physiological activity as that of the heart muscle cells *in vitro* is the fact that in certain lower forms of life a small bit of tissue or even a single cell, may develop in culture into a whole organism, demonstrating that the capacity of morphogenesis is retained in these isolated somatic cells. H. V. Wilson has shown that in coelenterates and sponges complete new individuals may develop *in vitro* from isolated cells taken from adult animals. By squeezing small bits of these animals through bolting cloth he was able to separate small groups of cells or even single cells. In culture these would grow into small masses of cells which would then differentiate slowly into the normal form of the complete organism. Figure 5 shows an example of this taken from Wilson's work.

It was early demonstrated by Carrel and Burrows that the life of the tissues *in vitro* which varied in different experiments from 5 to 20 days could be prolonged by a process of successive transfers of the culture to an indefinite period. Cells which were nearing the end of their life and growth in one culture need only be transferred to a new culture medium to keep on growing and multiplying. Dr. and Mrs. Warren H. Lewis made the important discovery that tissues of the chick embryo could be cultivated outside the body in purely inorganic solutions, such as sodium chloride, Ringer's solution, Locke's solution, etc. No growth in these inorganic cultures took place without sodium chloride. Growth was prolonged and increased by adding calcium and potassium. If maltose or dextrose, or protein decomposition products were added proliferation of the cells increased.

By the method of transfer to fresh nutrient media Carrel has been able to keep cultures of tissue from the heart of the chick embryo alive for a long period of years. In a letter recently received he says: "The strain of connective tissue obtained from a piece of chick heart

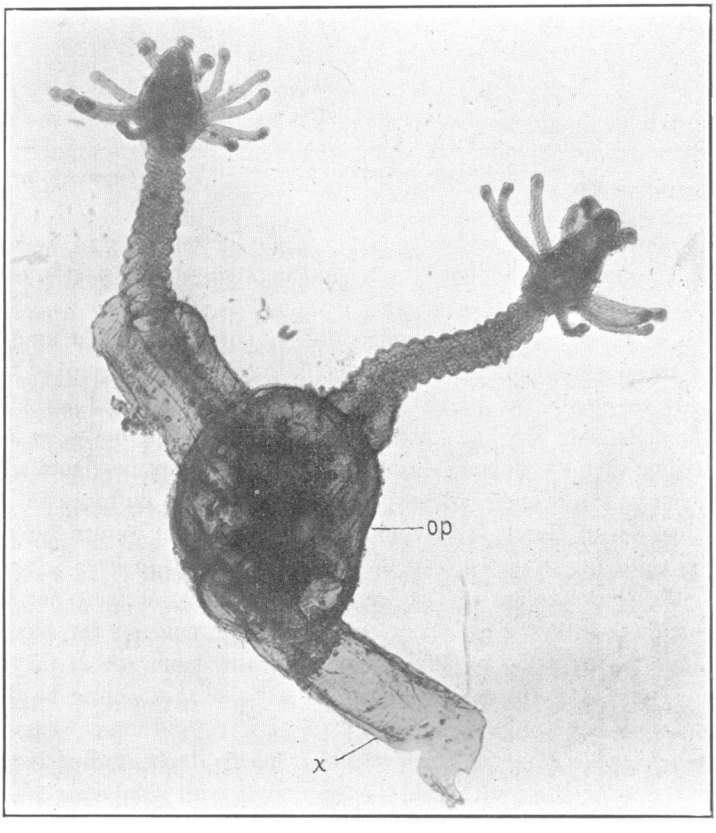

FIG. 5. PENNARIA. Restitution mass six days old, completely metamorphosed, with developed hydranths. Op. perisarc of original mass; x, perisarc of outgrowth adherent to glass. (From Wilson.)

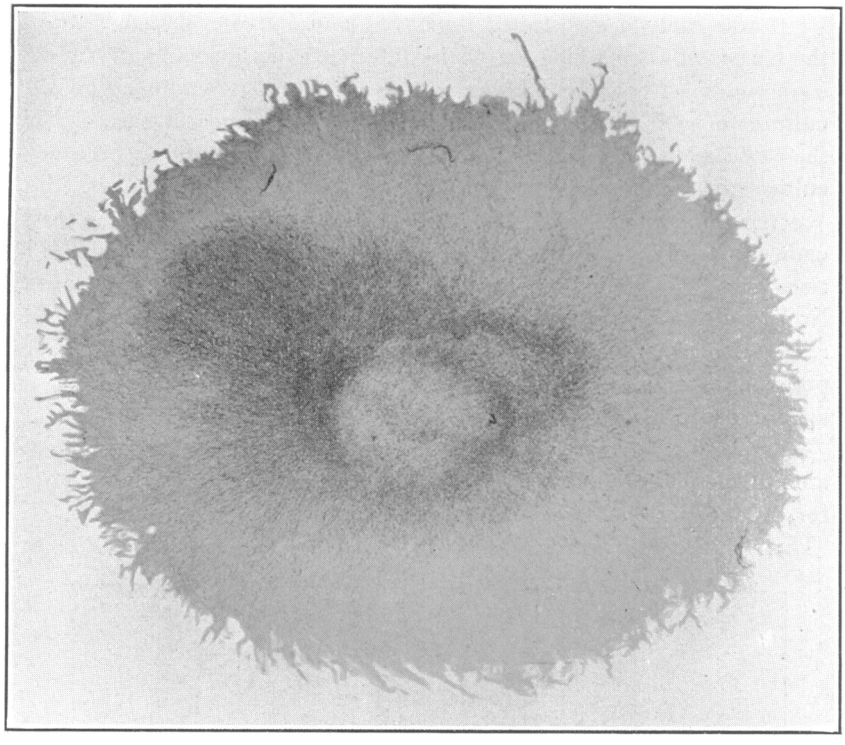

FIG. 6. CULTURE OF OLD STRAIN OF CONNECTIVE TISSUE. 1614 passage. 8 years and 8 months old, lacking 2 days. 48 hours' growth. x20. (Ebeling.)

is still alive and will be *nine years old* the seventeenth of January, 1921." Figure 6 is a photograph showing the present condition of this culture.

This is indeed a remarkable result. It completes the demonstration of the potential immortality of somatic cells, when removed from the body to conditions which permit of their continued existence. Somatic cells have lived and are still living outside the body for a far longer time than the normal duration of life of the species from which they came. I think the present extent of Carrel's cultures in time fully disposes of Harrison's criticism to the effect that we are "not justified in referring to the cells as potentially immortal or even in speaking of the prolongation of life by artificial means, at least not until we are able to keep the cellular elements alive in cultures for a period exceeding the duration of life of the organism from which they are taken. There is at present no reason to suppose this cannot be done, but it simply has not been done as yet." I have had many years' experience with the domestic fowl, and have particularly studied its normal duration of life, and discussed the matter with competent observers of poultry. I am quite sure that for most breeds of domestic poultry

the normal average expectation of life *at birth* is not substantially more than two years. For the longest lived races we know this normal average expectation of life cannot be over four years. I have never been able to keep a Barred Plymouth Rock alive more than seven years. There are on record instances of fowls living to as many as 20 years of age. But these are wholly exceptional instances, unquestionably far rarer than the occurrence of centenarians among human beings. There can be no question that the nine years of life of Carrel's culture has removed whatever validity may have originally inhered in Harrison's point. And further the culture is just as vigorous in its growth today as it ever was, and gives every indication of being able to go on indefinitely, for 20 or 40, or any desired number of years.

The potential immortality of somatic cells has been logically just as fully demonstrated in another way as it has by these tissue cultures. I fully agree with Leo Loeb when he says that the proof of potential immortality "can just as well be supplied by serial transplantation of tissues in the living body; but we can, I believe, go further, and state that as far as such potential immortality of tissues can be proved, the proof has already been given through the long-continued, apparently endless serial transplantation of tumors. Now tumor cells are merely ordinary somatic cells living under special conditions; and we may, therefore, conclude that, in the same sense as protozoa and germ cells, also, certain ordinary mammalian somatic cells possess a potential immortality." Loeb first announced this important conclusion nineteen years ago. To him unquestionably belongs the credit for first perceiving that death was not a necessary inherent consequence of life in the somatic cell, and demonstrating by actual experiments that somatic cells could, under certain conditions, go on living indefinitely.

Before turning to the next phase of our discussion let us summarize the ground we have covered up to this point. We have seen that by appropriate control of conditions it is possible to prolong the life of cells and tissues far beyond the limits of longevity to which they would attain if they remained in the multicellular body from which they came. This is true of a wide variety of cells and tissues differentiated in various ways. Indeed, the range of facts which have been ascertained by experimental work in this field probably warrants the conclusion that this potential longevity inheres in most of the different kinds of cells of the metazoan body, except those which are extremely differentiated for particular functions. To bring this potential immortality to actuality requires, of course, special conditions in each particular case. Many of these special conditions have already been discovered for particular tissues and particular animals. Doubtless, in the future many more will be worked out. We have furthermore seen that in certain cases the physico-chemical nature of the conditions necessary to insure the continuance of life has been definitely worked

out and is well understood. Again this warrants the expectation that, with more extended and penetrating investigations in a field of research which is really just at its beginning, we shall understand the physics and chemistry of prolongation of life of cells and tissues in a great many cases where now we know nothing about it.

One further point and we shall have done with this phase of our discussion. The experimental culture of cells and tissues *in vitro* has now covered practically all the *essential* tissue elements of the metazoan body, even including the most highly differentiated of those tissues. Nerve cells, muscle cells, heart muscle cells, spleen cells, connective tissue cells, epithelial cells from various locations in the body, kidney cells, and others have all been successfully cultivated *in vitro*. We may fairly say, I believe, that the potential immortality of all the essential cellular elements of the body either has been fully demonstrated, or else has been carried far enough to make the probability very great that properly conducted experiments would demonstrate the continuance of the life of these cells in culture to any definite extent. It is not to be expected, of course, that such tissues as hair, or nails, would be capable of independent life, but these are essentially unimportant tissues in the animal economy as compared with those of the heart, the nervous system, the kidneys, etc. What I am leading to is the broad generalization, perhaps not completely demonstrated yet, but having regard to Leo Loeb's work, so near it as to make little risk inhere in predicting the final outcome, *that all the essential tissues of the metazoan body are potentially immortal.* The reason that they are not actually immortal, and that multicellular animals do not live forever, is that in the differentiation and specialization of function of cells and tissues in the body as a whole, any individual part does not find the conditions necessary for its continued existence. In the body any part is dependent for the necessities of its existence, as for example nutritive material, upon other parts, or put in another way, upon the organization of the body *as a whole. It is the differentiation and specialization of function of the mutually dependent aggregate of cells and tissues which constitutes the metazoan body which brings about death, and not any inherent or inevitable mortal process in the individual cells themselves.*

3. Senescence

A careful and unprejudiced examination will suffice to convince anyone of open mind, I think, that much of the literature on senescence is really of no fundamental importance, because it has unwittingly reversed the true sequential order of the causal nexus. If cells of nearly every sort are capable, under appropriate conditions of living indefinitely in undiminished vigor, and cytological normality, there is little

ground for postulating that the observed senescent changes in these cells while in the body, such as those described by Minot and others, are expressive of specific and inherent mortal processes going on in the cells, or that these cellular processes are the *cause* of senescence, as Minot has concluded. It would rather appear that these visible cytological changes are expressive of effects not causes, and that they are the effects of the organization of the body as a whole as a system of mutually dependent parts, and not of specific, inherent and inevitable cellular processes.

Cells in culture *in vitro*, as we have seen, do not grow old. We see none of the characteristic senescent changes in them. From these facts it is a logically cogent induction to infer that when cells show the characteristic senescent changes, which were discussed in the preceding paper, it is because they are reflecting in their morphology and physiology a consequence of their mutually dependent association in the body as a whole, and not any necessary progressive process inherent in themselves. In other words we may justifiably, in the light of our present knowledge as I believe, regard *senescence as an attribute of the multicellular body as a whole*, consequent upon its scheme of morphologic and dynamic organization. This attribute is reflected morphologically in the component cells. But it does not originate in the cells, nor does it ever occur in the cells when they are removed from the mutually dependent relationship of the organized body as a whole. In short senescence is not a primary attribute of the physiological economy of cells as such.

If this conception of the phenomenon of senescence is correct in its main features, as I believe it is, it shows the essential futility of attempting to investigate its causes by purely cytological methods. On the other hand, by clearing away the unessential elements, it indicates where research into the problem of causation of senescence may be profitable.